Nutrient stress does not cause retrograde transport of cytoplasmic tRNA to the nucleus in evolutionarily diverse organisms

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ABSTRACT Intracellular trafficking of tRNA was long thought to be a one-way trip from the site of biogenesis in the nucleus to the translation machinery in the cytoplasm. This view has recently been challenged, however, by the discovery that tRNA can move retrograde from the cytoplasm back to the nucleus in *Saccharomyces cerevisiae* and rat hepatoma H4IIE cells during nutrient stress and in *S. cerevisiae* after intron-containing pre-tRNAs are spliced in the cytoplasm. Contrary to studies reported, we present data suggesting that nutrient stress does not cause retrograde transport of cytoplasmic tRNAs to the nucleus in rat hepatoma H4IIE cells, human HeLa and HEK293 cells, and the yeasts *Kluyveromyces lactis* and *S. cerevisiae*. However, the efficiency of nuclear re-export of retrograded spliced tRNA was severely affected in *S. cerevisiae* and two other *Saccharomyces* species deprived of nutrient. Collectively, the data suggest that nutrient stress does not cause nuclear import of cytoplasmic tRNA; instead, nutrient stress specifically regulates nuclear re-export of retrograded spliced tRNAs but not nuclear export of tRNAs made from intronless pre-tRNAs in *Saccharomyces* species. Furthermore, we provide evidence suggesting that Mtr10p and the Gsp1pGTP/Gsp-1pGDP cycle are not involved in nuclear tRNA import in *S. cerevisiae* during nutrient stress.

INTRODUCTION

Eukaryotic tRNAs are synthesized by RNA polymerase III as precursor molecules that undergo extensive processing to become mature tRNAs. For instance, maturation of precursor tRNAs involve trimming of the 5′ and 3′ ends, base modifications, addition of the CCA trinucleotide to the 3′ ends, and, for certain tRNA species, removal of an intron. For intronless pre-tRNAs, this maturation process occurs entirely in the nucleus of mammalian, plant, and yeast cells. Maturation of intron-containing pre-tRNAs also occurs in the nucleus; however, in *Saccharomyces cerevisiae*, unlike in vertebrates and plants, intron-containing tRNAs lacking the 5′ and 3′ extensions are exported to the cytoplasm for removal of the intron, and the newly spliced tRNAs are returned to the nucleus for reasons that are

not understood. The retrograded tRNAs are then re-exported from the nucleus to the cytoplasm for use in protein synthesis (Hopper and Phizicky, 2003; Yoshihisa *et al.*, 2003, 2007; Paushkin *et al.*, 2004; Shaheen and Hopper, 2005; Takano *et al.*, 2005; Englert *et al.*, 2007). The surprising discovery of bidirectional transport of tRNA between the nucleus and cytoplasm in *S. cerevisiae* has opened up the possibility that other eukaryotes have the capacity to retrograde cytoplasmic tRNAs to the nucleus.

Retrograde transport of spliced tRNAs from the cytoplasm to the nucleus has been shown to occur by a constitutive process (Shaheen and Hopper, 2005; Takano *et al.*, 2005; Murthi *et al.*, 2010). However, retrograded spliced tRNAs were found to accumulate in the nucleus of *S. cerevisiae* deprived of glucose, nitrogen, amino acid, or P_i (Shaheen and Hopper, 2005; Hurto *et al.*, 2007; Whitney *et al.*, 2007). Nuclear accumulation of tRNAs made from intronless pre-tRNAs was also detected in *S. cerevisiae* in response to nutrient stress (Shaheen and Hopper, 2005). Moreover, accumulation of a tRNA derived from an intronless precursor was observed in the nucleus of rat hepatoma H4IIE cells starved of amino acids (Shaheen *et al.*, 2007). These studies established that mammalian and *S. cerevisiae* cells retrograde cytoplasmic tRNAs to the nucleus in response to nutrient availability. Genetic studies in *S. cerevisiae*

suggest that the protein kinase A (PKA) and SNF1 signal transduction pathways may be regulating the tRNA retrograde process in the absence of glucose, whereas the rapamycin-sensitive serine/threonine kinase TOR is involved when cells are deprived of amino acids (Whitney et al., 2007). These findings led to the suggestion that nuclear import of tRNAs in *S. cerevisiae* and mammalian cells may play an important role in nutrient-related regulation of gene expression and cell growth (Shaheen et al., 2007; Whitney et al., 2007). However, it is not known whether nuclear import of cytoplasmic tRNAs is generally involved in nutrient regulation of gene expression in eukaryotes.

To assess whether retrograde movement of tRNA from the cytoplasm to the nucleus in response to nutrient stress is conserved among evolutionarily diverse organisms, we investigated the effect of nutrient deprivation on nuclear-cytoplasmic trafficking of tRNAs in human (HEK293 and HeLa) and rat (rat hepatoma H4IIE) cell lines, *Kluyveromyces lactis*, and three species of *Saccharomyces* (*S. cerevisiae*, *S. paradoxus*, and *S. bayanus*). The results show that exposure of *S. cerevisiae*, *S. paradoxus*, and *S. bayanus* to nutrient stress resulted in nuclear accumulation of spliced tRNA. In contrast, nutrient starvation of mammalian cells and *K. lactis* did not cause nuclear accumulation of spliced tRNAs. This finding suggests that retrograde transport of spliced tRNAs made from intron-containing precursors from the cytoplasm to the nucleus may be limited to the genus *Saccharomyces*. In contrast to previous studies, nuclear accumulation of tRNAs made from intronless pre-tRNAs was not observed in *S. cerevisiae* subjected to nutrient stress. Similarly, nutrient starvation of mammalian cells did not affect nuclear-cytoplasmic trafficking of tRNAs made from intronless precursors. Taken together, the results suggest that mammalian and yeast cells do not retrograde cytoplasmic tRNAs to the nucleus in response to nutrient stress. Moreover, the data suggest that nutrient stress specifically regulates nuclear re-export of retrograded spliced tRNAs but not nuclear export of mature tRNAs derived from intronless precursors in *Saccharomyces* species. This conclusion is consistent with recent studies that show that the pathways used for nuclear re-export of retrograded spliced tRNAs, but not those that facilitate nuclear export of tRNAs made from intronless precursors, are regulated by the TOR signaling pathway in *S. cerevisiae* (Pierce et al., 2010). Furthermore, in contrast to previous studies, we provide evidence suggesting that Mtr10p and the RanGTP/RanGDP cycle do not play a role in nuclear import of cytoplasmic spliced tRNAs in *S. cerevisiae* during nutrient stress (Shaheen and Hopper, 2005).

RESULTS AND DISCUSSION
Human HeLa and HEK293 and rat hepatoma H4IIE cells deprived of amino acids do not accumulate cytoplasmic tRNA in the nucleus

Rat hepatoma H4IIE cells have been shown to accumulate tRNA in the nucleus upon amino acid starvation, leading to the proposal that, like *S. cerevisiae*, mammalian cells have the capacity to carry out nuclear import of cytoplasmic tRNAs in response to nutrient availability (Shaheen et al., 2007). We therefore tested whether human cells can also retrograde transport tRNAs from the cytoplasm to the nucleus in response to nutrient stress. For this analysis, the effect of amino acid deprivation on nuclear-cytoplasmic distribution of tRNA in HeLa and HEK293 human cell lines was compared with that in rat hepatoma H4IIE cells.

Previous studies have demonstrated that amino acid starvation of rat hepatoma H4IIE cells resulted in reduced phosphorylation of the eukaryotic initiation factor 4E binding protein (4E-BP1), which was detected as a faster migrating species by SDS–PAGE and West-
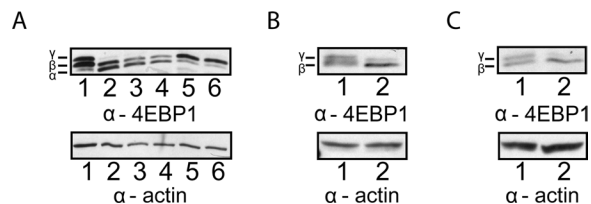

FIGURE 1: Amino acid starvation affects phosphorylation of 4E-BP1 in rat hepatoma H4IIE, HeLa, and HEK293 cells. Lysates were obtained from H4IIE cells (A) incubated for 2.5 h in complete DMEM (lane 1), starved of serum, and then incubated in DMEM without Lys, Arg, Leu, and Gln for 2.5 h (lane 2), serum-free DMEM lacking Lys, Arg, Leu, and Gln (lane 3), serum-free DMEM without any amino acids (lane 4), PBS containing glucose (lane 5) or Ham's F-12 nutrient media (lane 6), and from HeLa (B) or HEK293 (C) cells incubated for 2.5 h in complete DMEM (lane 1) or serum-free DMEM without any amino acids (lane 2). An aliquot of lysate (20 µg protein) was separated by SDS–PAGE on a 15% polyacrylamide gel, and Western blot analysis was used to detect 4E-BP1 (top) or actin (bottom).

ern blot analyses (Shaheen et al., 2007). Consequently, the phosphorylation state of 4E-BP1 was used to verify that amino acid starvation has the expected effect on HeLa, HEK293, and rat hepatoma H4IIE cells (Figure 1). Western blot analysis of cell extract prepared from rat hepatoma H4IIE cells (Figure 1A, top row) grown in complete media (Figure 1A, lane 1) detected the γ- and β-, and to a lesser extent, the α-phosphorylated forms of 4E-BP1, the former two being the major phosphorylated forms of 4E-BP1 reported for unstarved cells (Shaheen et al., 2007). In contrast, only the β- and α-phosphorylated forms of 4E-BP1 were detected in rat hepatoma H4IIE cells starved of serum and then for four essential amino acids (Lys, Arg, Leu, and Gln) in DMEM (Figure 1A, lane 2), starved of the four essential amino acids in DMEM (Figure 1A, lane 3), or starved of all amino acids in DMEM (Figure 1A, lane 4). In cell extracts prepared from rat hepatoma H4IIE cells starved of all amino acids in phosphate-buffered saline (PBS) containing glucose (Figure 1A, lane 5), or starved of Met, Gln, Lys, and Leu in Hams F-12 nutrient media (Figure 1A, lane 6), the β-phosphorylated form of 4E-BP1 was primarily detected. Western blot analysis of actin shows that essentially the same amount of cell extract was analyzed (Figure 1A, bottom row). The γ- and β-phosphorylated forms of 4E-BP1 were also detected in unstarved HeLa (Figure 1B, top row, lane 1) and HEK293 (Figure 1C, top row, lane 1) cells. However, the β-phosphorylated form of 4E-BP1 was the main species observed in these cells when they are starved of all amino acids in DMEM (Figure 1, B and C, lane 2). Western blot analysis of actin confirmed that the same amount of cell extract was analyzed (Figure 1, B and C, bottom row). The data indicate that rat hepatoma H4IIE cells respond, as expected, to amino acid deprivation irrespective of the media used and that amino acid starvation also has a similar effect on human cells.

The effect of amino acid starvation on the nuclear-cytoplasmic distribution of mature tRNA[Lys], a tRNA derived from intronless pre-tRNA, was investigated in rat hepatoma H4IIE, HeLa, and HEK293 cells. Mature tRNA[Lys] was monitored by fluorescence in situ hybridization (FISH), since it was previously used to demonstrate that rat hepatoma H4IIE cells retrograde cytoplasmic tRNA to the nucleus in response to amino acid starvation (Shaheen et al., 2007). However, to verify that the FISH conditions used in this study are capable of detecting nuclear retention of endogenous tRNAs in mammalian cells, the cellular location of tRNA[His] was monitored by FISH in HeLa, rat hepatoma H4IIE, and HEK293 cells incubated in DMEM lacking histidine and containing histidinol, an analogue of histidine that has
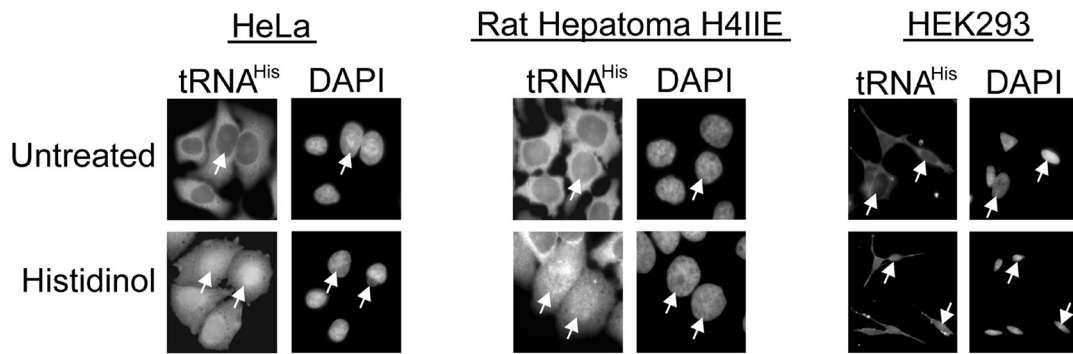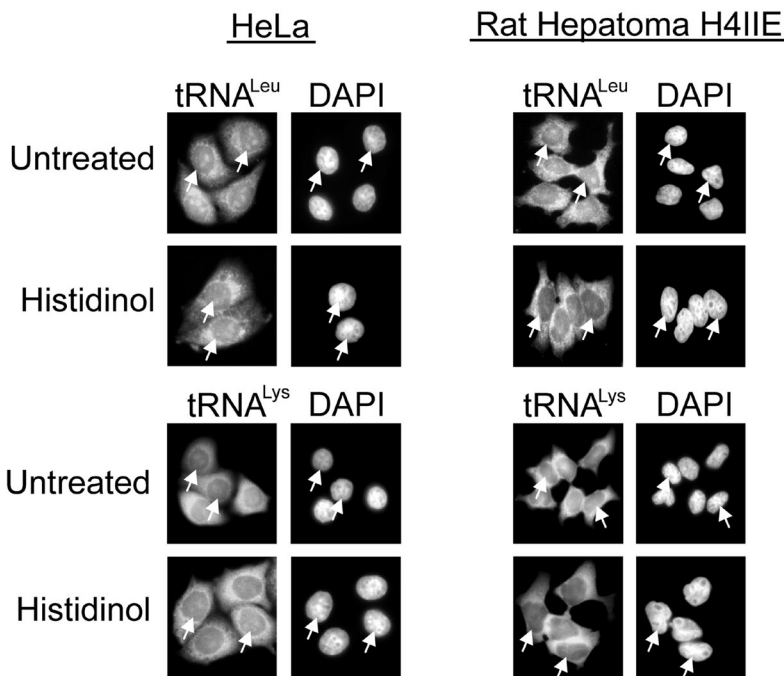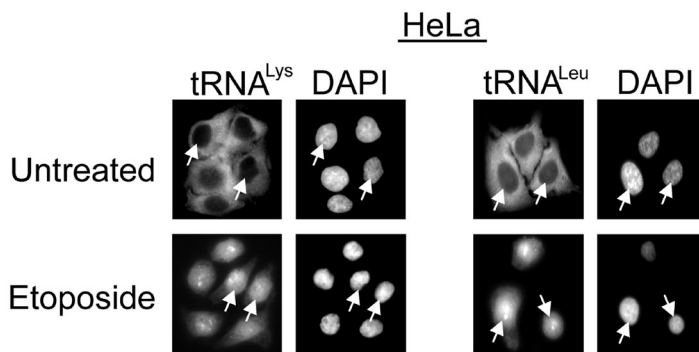
FIGURE 2: FISH detects nuclear retention of endogenous tRNAs in mammalian cells. (A, B) Inhibition of the aminoacylation activity of the histidinyl-tRNA synthetase in mammalian cells reduces the efficiency of nuclear export of tRNA^{His} but not tRNA^{Leu} and tRNA^{Lys}. HeLa, HEK293, and H4IIE cells were grown on coverslips overnight. The cells were washed and placed in serum-free complete DMEM or DMEM lacking histidine and containing 2 mM histidinol for 6 h. Following the treatment, the cells were washed with 1× PBS, fixed, and processed for FISH to detect the cellular location of tRNA^{His} (A), tRNA^{Leu}, and tRNA^{Lys} (B). (C) Etoposide treatment of HeLa cells causes nuclear retention of tRNA^{Lys} and tRNA^{Leu}. HeLa cells were incubated in serum-free DMEM alone (untreated) or containing 34 μM etoposide (etoposide) for 48 h. The cells were washed and fixed in 1× PBS containing 4% formaldehyde, and the distribution of tRNA^{Lys} and tRNA^{Leu} was monitored by FISH. The cells were DAPI stained to visualize the nucleus. The arrows indicate the location of nuclei.
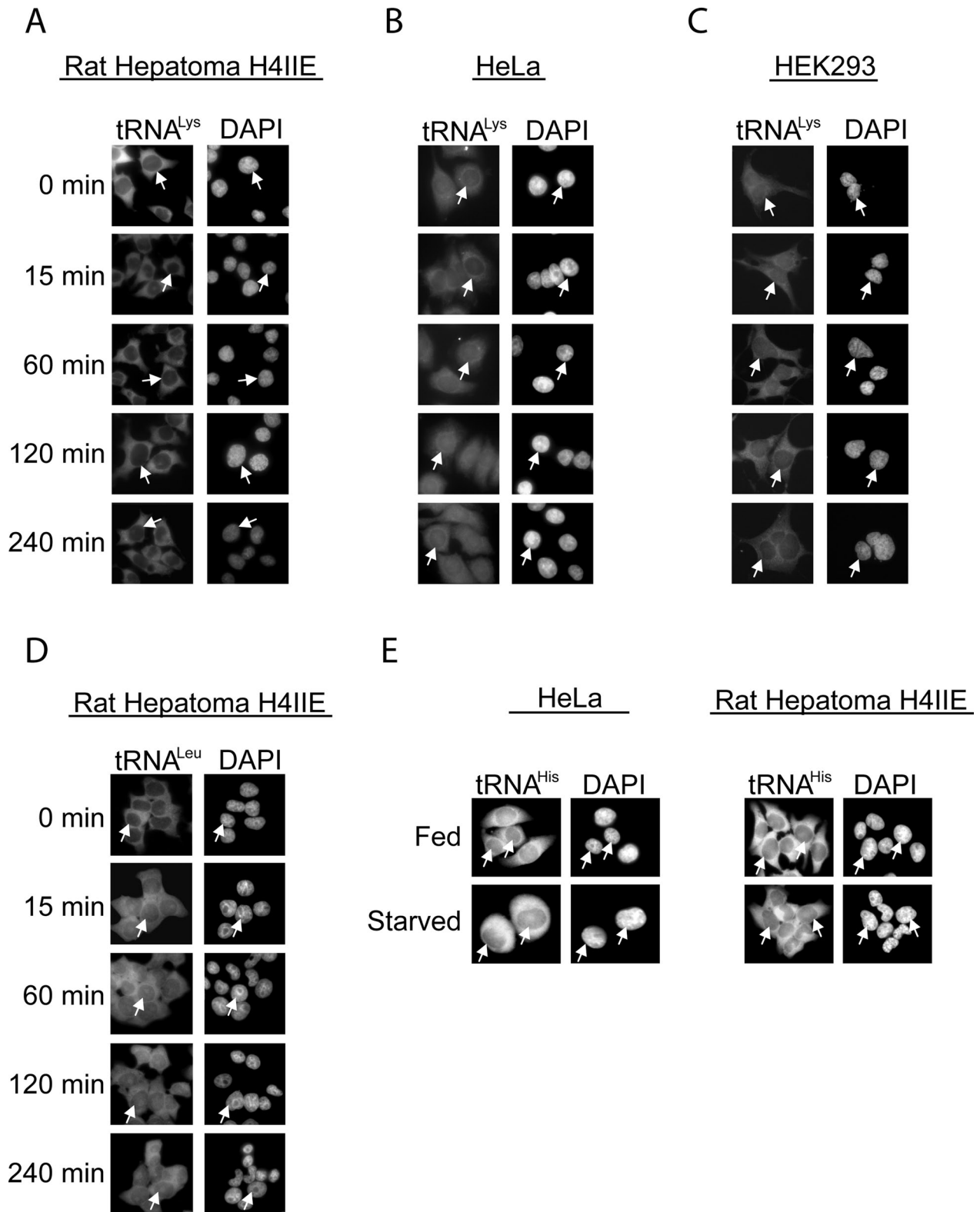
FIGURE 3: Amino acid starvation does not cause nuclear accumulation of tRNA in mammalian cells. Rat hepatoma H4IIE (A, D, E), HeLa (B, E), or HEK293 (C) cells were incubated in serum-free DMEM without amino acids for the times specified, and the nuclear-cytoplasmic distribution of tRNA^{Lys} (A–C), tRNA^{Leu} (D), and tRNA^{His} (E) was monitored by FISH. Nuclei were visualized by DAPI staining of the DNA. The arrows indicate the location of nuclei.

been shown to be a competitive inhibitor of the histidinyl–tRNA synthetase (Filetti and Rapoport, 1982) (Figure 2A). The analyses show that tRNA^{His} is retained in the nucleus of HeLa, rat hepatoma H4IIE,

and HEK293 cells treated with histidinol in the absence of histidine for 6 h. However, histidinol treatment did not affect nuclear export of tRNA^{Leu} or tRNA^{Lys} in HeLa and rat hepatoma H4IIE (Figure 2B),

indicating that histidinol treatment does not have a general effect on nuclear tRNA export. This is consistent with studies showing that the efficiency of nuclear export of tRNA^{Tyr} but not tRNA^{Met} in *Xenopus* oocytes is reduced considerably when the tyrosyl–tRNA synthetase activity is inhibited (Lund and Dahlberg, 1998).

Studies reported recently indicate that, during the early stages of apoptosis, induced by etoposide treatment, Ran is retained in the cytoplasm (Wong *et al.*, 2009). Mislocalization of Ran to the cytoplasm was shown to block nuclear import of nuclear localization signal (NLS)-containing proteins (Wong *et al.*, 2009). Xpo-t is the major nuclear export receptor for tRNAs in mammalian cells, and it is fully established that Xpo-t binding to tRNA is facilitated by interaction with RanGTP in the nucleus (Arts *et al.*, 1998; Kutay *et al.*, 1998). Thus, mislocalization of Ran to the cytoplasm by etoposide treatment was used to establish that nuclear retention of tRNA^{Lys} and tRNA^{Leu} is detectable by FISH (Chafe and Mangroo, 2010) (Figure 2C). Both tRNA^{Lys} and tRNA^{Leu} were found to accumulate in the nucleus in HeLa cells treated with etoposide. In contrast, nuclear accumulation of the tRNAs was not observed in untreated cells. These results show that inhibition of nuclear tRNA export by lack of RanGTP in the nucleus causes nuclear accumulation of the tRNAs and demonstrate that the FISH conditions and oligonucleotides used in this study are clearly capable of detecting nuclear accumulation of the tRNAs in mammalian cells. Furthermore, Northern blot analyses also show that the oligonucleotides detect tRNA^{Lys} in total RNA isolated from rat hepatoma H4IIE and HeLa cells, and tRNA^{Leu} in total RNA isolated from rat hepatoma H4IIE cells (Supplemental Figure S1).

Contrary to previous reports, our analyses (Figure 3) show that starvation of rat hepatoma H4IIE (Figure 3A), HeLa (Figure 3B), and HEK293 (Figure 3C) cells of amino acids in DMEM over a 240-min period did not change the nuclear-cytoplasmic distribution of tRNA^{Lys}. Similarly, starvation of rat hepatoma H4IIE cells of amino acids in PBS containing glucose, serum followed by deprivation of Lys, Arg, Leu, and Gln in DMEM, DMEM lacking Lys, Arg, Leu, and Gln, or Ham's F12 nutrient media lacking Met, Lys, Leu, and Gln did not result in nuclear retention of tRNA^{Lys} (data not shown). Furthermore, amino acid starvation of rat hepatoma H4IIE over a 240-min period did not result in nuclear accumulation of mature tRNA^{Leu}, which is derived from intron-containing pre-tRNA (Figure 3D) and tRNA^{His} (Figure 3E). Nuclear retention of tRNA^{His} was also not observed in HeLa cells starved of amino acids (Figure 3E). Thus, despite observing an effect on phosphorylation of 4E-BP1 when rat hepatoma H4IIE, HeLa, or HEK293 cells are starved of amino acids and on nuclear tRNA export in cells treated with etoposide or histidinol, we could not detect nuclear accumulation of mature cytoplasmic tRNA^{Lys}, tRNA^{Leu}, and tRNA^{His} by FISH. Likewise, glucose starvation did not cause nuclear accumulation of tRNA^{Lys} in rat hepatoma H4IIE and HeLa cells (Supplemental Figure S2). In addition, nuclear retention of tRNA^{Lys} was not observed during amino acid starvation of rat hepatoma H4IIE cells when the amount of the 5′-Cy3-labeled oligonucleotide was increased from 5 to 40 pmol (Supplemental Figure S3, left). However, a signal was observed in the nucleus of amino acid–starved rat hepatoma H4IIE cells when 10 or 40 pmol of the oligonucleotide labeled with an undefined number of digoxigenin (DIG)-UTP was used for hybridization and fluorescein isothiocyanate–tagged anti-DIG antibodies were used for detection (Supplemental Figure S3, right) (Shaheen *et al.*, 2007). However, the abundance of this signal is considerably lower than that reported previously (Shaheen *et al.*, 2007). Thus, in contrast to previous studies (Shaheen *et al.*, 2007), our data collectively suggest that nutrient deprivation does not result in retrograde transport of cytoplasmic tRNAs to the nucleus or in a block in nuclear export of tRNA in mammalian cells.

Sodium azide treatment has no effect on nuclear-cytoplasmic tRNA trafficking in rat hepatoma H4IIE cells

Nuclear accumulation of tRNA^{Lys} was observed in rat hepatoma H4IIE cells treated with 10 mM sodium azide for 150 min (Shaheen *et al.*, 2007). Therefore, we tested whether sodium azide treatment of rat hepatoma H4IIE cells causes nuclear accumulation of tRNA^{Lys}. As shown previously (Shaheen *et al.*, 2007), only the α-phosphorylated form of 4E-BP1 was detected in cell extract prepared from cells treated with 10 mM sodium azide for 150 min (Figure 4A, top row, lane 2), whereas, as expected, the γ-, β-, and to a
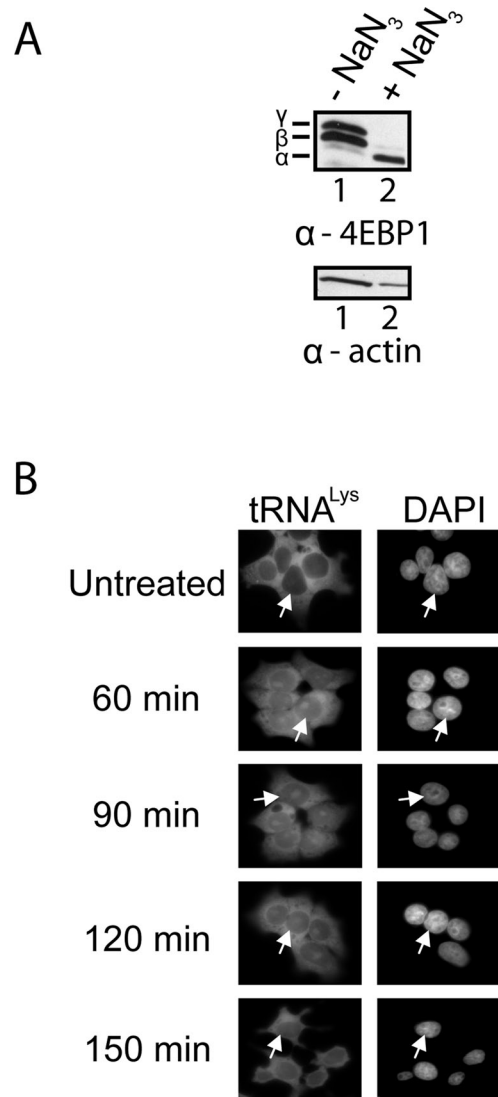
FIGURE 4: Sodium azide treatment affects phosphorylation of 4E-BP1 but not the distribution of tRNA in rat hepatoma H4IIE cells. (A) Lysates were prepared from cells incubated for 2.5 h in DMEM without serum in the absence (lane 1) or presence (lane 2) of 10 mM NaN$_3$, and an aliquot of lysate (20 μg protein) was separated by SDS–PAGE on a 15% polyacrylamide gel. Western blot analysis was used to detect 4E-BP1 (upper) or actin (bottom). (B) Nuclear-cytoplasmic distribution of tRNA^{Lys} was monitored by FISH in cells incubated with 10 mM NaN$_3$ for the times indicated. The arrow indicates the location of the nucleus.

lesser extent, the α-phosphorylated forms of the protein (Figure 4A, lane 1) were detected in untreated cells. Western blot analysis of the actin level showed that a smaller amount of cell extract from treated cells was used (Figure 4A, bottom row, lane 2). In contrast to results reported (Shaheen *et al.*, 2007), treatment of rat hepatoma H4IIE cells with sodium azide over a 150-min period did not affect nuclear-cytoplasmic trafficking of mature tRNA^{Lys} (Figure 4B). Taken together, the data obtained by our studies suggest that nuclear-cytoplasmic trafficking of tRNAs in mammalian cells is not affected by nutrient stress or inhibition of ATP production.

Amino acid starvation affects nuclear-cytoplasmic trafficking of spliced tRNA in several species of *Saccharomyces* but not in *K. lactis*

Our finding that nuclear-cytoplasmic tRNA trafficking in mammalian cells is not affected by nutrient stress suggests that nutrient-related nuclear import of cytoplasmic tRNAs is either unique to *S. cerevisiae* alone or is a characteristic of all yeasts. To test whether nuclear import of tRNA in response to nutrient stress is specific for yeast or perhaps just to species of *Saccharomyces*, we investigated the effect of nutrient stress on nuclear-cytoplasmic trafficking of tRNA^{Tyr} in *K. lactis* and two closely related species of *Saccharomyces*: *S. bayanus* and *S. paradoxus* (Figure 5). Nuclear accumulation of

tRNA^{Tyr} was observed by FISH in *S. cerevisiae* (Figure 5A) and both *S. paradoxus* (Figure 5B) and *S. bayanus* (Figure 5C) cells starved of amino acids or glucose (Supplemental Figure S4). However, neither amino acid (Figure 5D) nor glucose (Supplemental Figure S4) deprivation of *K. lactis* had an effect on the nuclear-cytoplasmic distribution of tRNA^{Tyr}. Furthermore, the oligonucleotide probe used detects tRNA^{Tyr} by Northern blot analysis of total RNA isolated from *K. lactis* (Figure 5E). In addition, starving *K. lactis*, which is a leucine auxotroph, of leucine only for 4 h resulted in nuclear accumulation of tRNA^{Leu} but not tRNA^{Tyr} (Figure 5F), which is consistent with a reduction in the efficiency of nuclear export of tRNA^{Leu} by lack of aminoacylation. Taken together, the data suggest that nuclear import of cytoplasmic tRNA in response to nutrient stress may be limited to yeasts of the genus *Saccharomyces*. This conclusion is consistent with the observation that nuclear import of cytoplasmic tRNAs does not occur in *Schizosaccharomyces pombe* starved of amino acids, nitrogen, or glucose (data not shown).

Amino acid starvation does not cause nuclear import of cytoplasmic tRNAs made from intronless precursors in *S. cerevisiae*

Mature tRNAs derived from intronless pre-tRNAs have been shown to accumulate in the nucleus of *S. cerevisiae* starved of amino acids
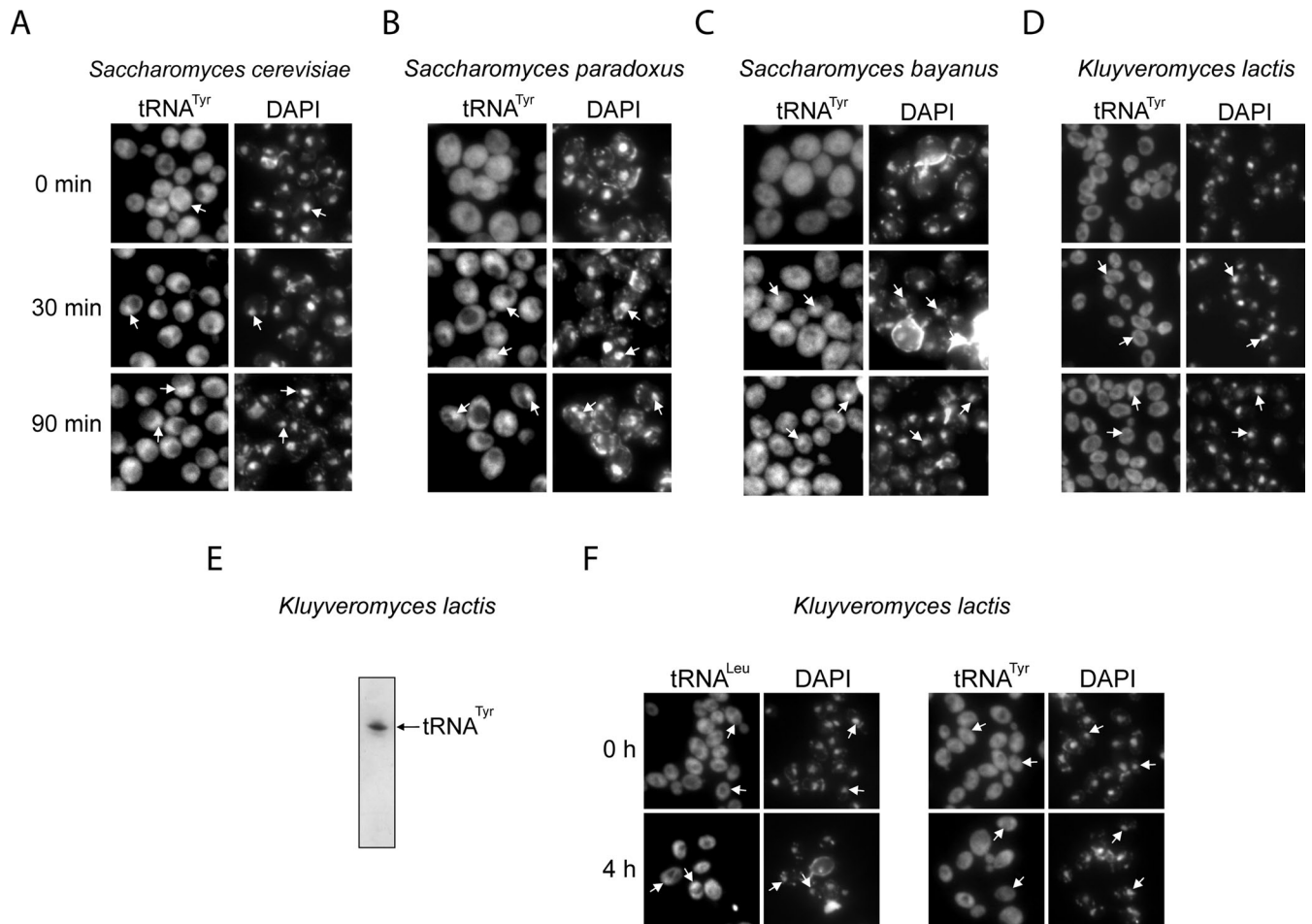

FIGURE 5: Amino acid deprivation causes nuclear accumulation of spliced tRNAs in *S. cerevisiae*, *S. paradoxus*, and *S. bayanus* but not in *K. lactis*. *S. cerevisiae* (A), *S. paradoxus* (B), *S. bayanus* (C), and *K. lactis* (D) were starved of amino acids in minimal medium containing glucose for the times indicated. The location of tRNA^{Tyr} was detected by FISH. The arrows indicate the location of nuclei. Northern blot analysis was used to confirm that the oligonucleotide detects tRNA^{Tyr} in total RNA isolated from *K. lactis* (E). Nuclear retention of tRNA^{Leu} but not tRNA^{Tyr} was observed when *K. lactis*, which is auxotrophic for leucine, was starved of leucine for 4 h (F).
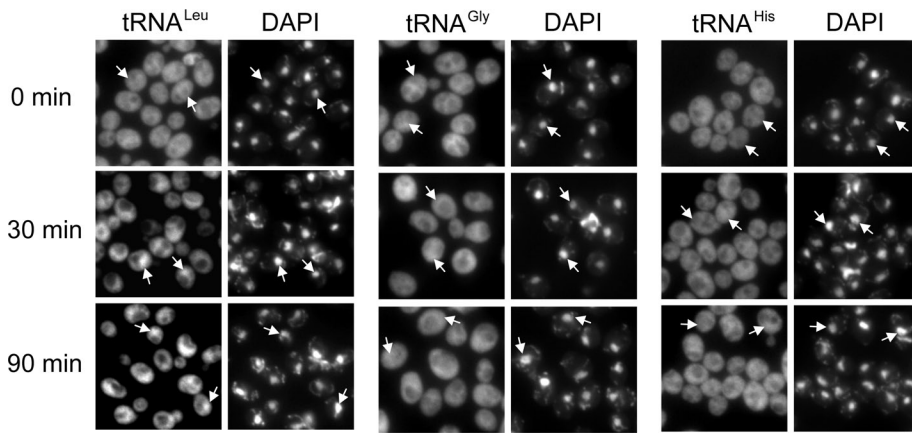
FIGURE 6: Amino acid starvation for a short time period does not affect the nuclear-cytoplasmic distribution of mature tRNAs made from intronless pre-tRNAs in *S. cerevisiae*. The cells were starved of amino acids in minimal medium containing glucose for the times indicated. The location of tRNA[Leu], tRNA[His], and tRNA[Gly] was detected by FISH. The arrows indicate the location of nuclei.

or nitrogen, which is also consistent with the conclusion that *S. cerevisiae* retrogrades cytoplasmic tRNAs to the nucleus in response to nutrient stress (Shaheen and Hopper, 2005; Whitney et al., 2007). These findings combined with the ability of several *Saccharomyces* species, including *cerevisiae*, to transport spliced tRNAs in the cytoplasm back to the nucleus suggest that nuclear import of cytoplasmic tRNAs in response to nutrient availability may be limited to yeasts of the genus *Saccharomyces*. However, it was recently reported that neither nitrogen nor amino acid starvation causes nuclear accumulation of tRNAs derived from intronless pre-tRNAs (Eswara et al., 2009; Pierce et al., 2010). This finding led to the suggestion that amino acid or nitrogen starvation does not cause nuclear import of cytoplasmic tRNAs in *S. cerevisiae*. Moreover, it was proposed that nuclear re-export of retrograded spliced tRNAs, but not nuclear export of tRNAs made from intronless precursors, is regulated by nutrient stress (Eswara et al., 2009; Pierce et al., 2010). This is consistent with studies showing that nuclear import of spliced

tRNA 2 in *S. cerevisiae* occurs by a constitutive process (Shaheen and Hopper, 2005; Murthi et al., 2010). Nevertheless, to investigate whether amino acid deprivation of *S. cerevisiae* results in nuclear accumulation of cytoplasmic tRNAs synthesized from intronless pre-tRNAs in the nucleus, FISH was used to monitor the nuclear-cytoplasmic distribution of tRNA[Gly] and tRNA[His], which are two tRNAs made from intronless precursors. The FISH conditions and concentration of probe used are identical to those used to detect nuclear accumulation of tRNA[Tyr] in *S. cerevisiae* deprived of amino acids. As observed before for tRNA[Tyr], amino acid starvation of *S. cerevisiae* for 30 min resulted in nuclear accumulation of tRNA[Leu], another tRNA made from intron-containing pre-tRNA (Figure 6) (Whitney et al., 2007). However, in contrast to previous studies (Shaheen and Hopper, 2005), we found that the nuclear-cytoplasmic distribution of tRNA[Gly] and tRNA[His] was not affected by amino acid starvation over a 90-min period (Figure 6), which is in accordance with other studies (Eswara et al., 2009). As expected, nuclear accumulation of both tRNA[Tyr] and tRNA[Gly] was observed in *msn5 los1* (Supplemental Figure S5), a strain that lacks the function of the nuclear tRNA export receptors Msn5p and Los1p (Takano et al., 2005). Msn5p facilitates nuclear re-export of retrograded spliced tRNAs (Eswara et al., 2009; Murthi et al., 2010), whereas Los1p facilitates both nuclear export of retrograded spliced tRNAs (Murthi et al., 2010) and tRNAs made from intronless pre-tRNAs.

The majority of tRNAs in *S. cerevisiae* is encoded by either intron-containing or intronless genes. However, several tRNA families, such as tRNA[Ile], in *S. cerevisiae* are encoded by both intronless and intron-containing genes. Moreover, previous studies have indicated that both tRNA[Ile] species can be detected by FISH analysis in *S. cerevisiae*, demonstrating that both genes are
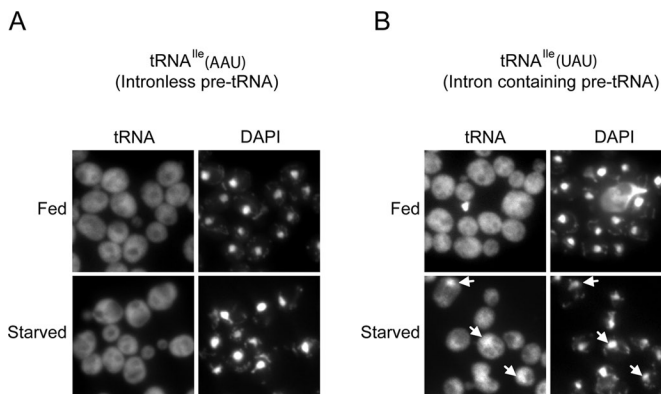
A

B

FIGURE 7: Amino acid starvation causes nuclear retention of tRNA[Ile(UAU)] derived from an intron-containing precursor, but not tRNA[Ile(AAU)] made from an intronless pre-tRNA. *S. cerevisiae* cells were starved of amino acids in synthetic medium containing glucose for 1 h, and the cellular distribution of mature tRNA[Ile(AAU)] (A) and tRNA[Ile(UAU)] (B) was monitored by FISH. The DNA was visualized by DAPI staining. Arrows indicate nuclear accumulated tRNAs that colocalize with DAPI staining.

| Species	Strain	Genotype
S. cerevisiae	BY4742	*MATα, his3Δ1 leu2 lys2 ura3*
	RS453	*MATα/MATa, ade2/ade2 his3/ his3 leu2/leu2 trp1/trp1 ura3/ ura3*
	mtr10	*MATα, ade2 his3 leu2 trp1 ura3 mtr10::HIS3*
	rna1–1	*MATa, rna1–1 rnh1::URA3 ura3–52 his7 his4 tyr1 ade1*
S. paradoxus	A4	Isolated from bark of *Quercus rubra* in Mont St-Hilaire, Quebec, Canada
S. bayanus	DBY17007	*MATa, hoΔ::KAN*
K. lactis	NK40	*MATα, ade1 ade2 leu2*
los1 msn5	BY4741 derivative	*MATa, los1::natMX msn5::kanMX his3Δ1 leu2 ura3*

TABLE 1: Yeast strains used in this study.
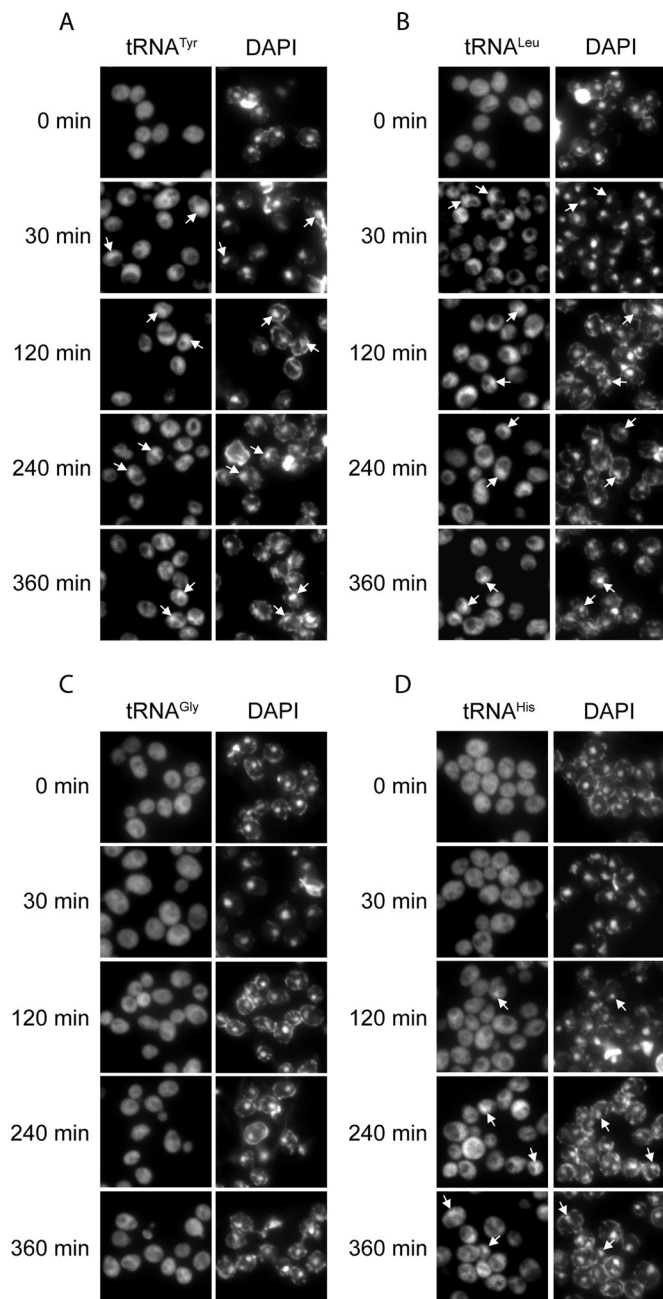
FIGURE 8: The onset of nuclear retention of tRNA^His in the histidine, leucine auxotrophic *S. cerevisiae* strain starved of amino acids occurs later than that for tRNA^Tyr and tRNA^Leu. *S. cerevisiae* was incubated in complete minimal medium lacking all amino acids over a 6-h period, and the cellular location of the mature forms of tRNA^Tyr, tRNA^Leu, tRNA^Gly, and tRNA^His was monitored by FISH. DAPI staining was used to visualize the nucleus.
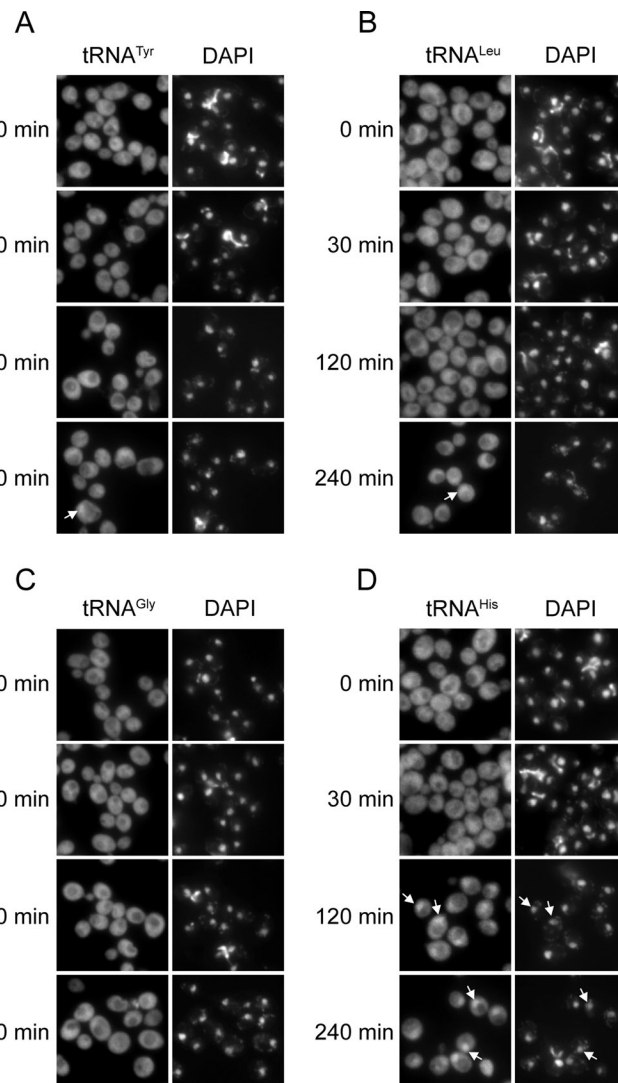

FIGURE 9: The onset of nuclear accumulation of tRNA^Tyr and tRNA^Leu in the histidine, leucine auxotrophic *S. cerevisiae* strain starved of histidine only occurs later than that for tRNA^His. *S. cerevisiae* was incubated in complete minimal medium lacking histidine over a 4-h period, and the cellular location of the mature forms of tRNA^Tyr, tRNA^Leu, tRNA^Gly, and tRNA^His was monitored by FISH. Nuclei were visualized by DAPI staining.

transcribed (Grosshans *et al.*, 2000). To verify that nutrient deprivation does not affect nuclear export of tRNAs made from intronless genes, nuclear export of tRNA^Ile(UAU) made from intron-containing pre-tRNA and tRNA^Ile(AAU) made from the intronless precursor were monitored during amino acid starvation of *S. cerevisiae* cells. For this analysis, the cells were grown in synthetic media containing glucose and lacking amino acids for 1 h, and FISH was used to monitor the cellular location of tRNA^Ile(AAU) (Figure 7A) and tRNA^Ile(UAU) (Figure 7B). The analyses show uniform distribution of both tRNA^Ile species in unstarved cells. However,

although the localization of tRNA^Ile(AAU) (Figure 7A), which is derived from an intronless pre-tRNA, remains unaffected by amino acid starvation, nuclear accumulation was observed for tRNA^Ile(UAU) (Figure 7B) made from the intron-containing precursor.

The *S. cerevisiae* strain used in this study is auxotrophic for histidine, lysine, and leucine (Table 1), and we have established that starvation of this strain of all amino acids for 30 min resulted in nuclear accumulation of tRNA^Tyr (Figure 5A), tRNA^Ile (UAU) (Figure 7B), and tRNA^Leu (Figure 6) made from intron-containing precursors, but not tRNA^Ile (AAU) (Figure 7A), tRNA^Gly, and tRNA^His made from intronless pre-tRNAs (Figure 6). Furthermore, starvation for 90 min also did not result in nuclear accumulation of tRNA^His or tRNA^Gly (Figure 6). These results are consistent with the suggestion that amino acid starvation does not cause nuclear import of cytoplasmic tRNAs derived from intronless pre-tRNAs. Nevertheless, it is anticipated that tRNA^His should accumulate in the nucleus if lack of aminoacylation of the tRNA in the nucleus reduces the efficiency of its
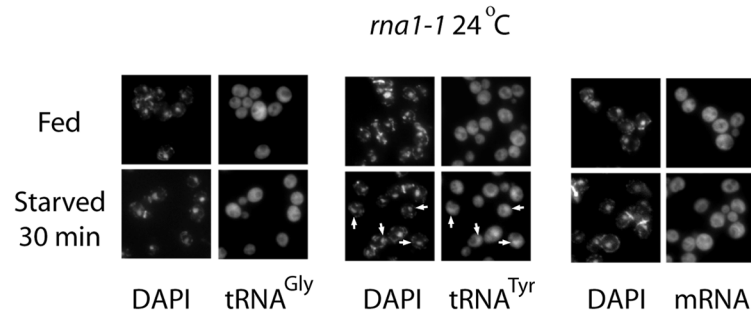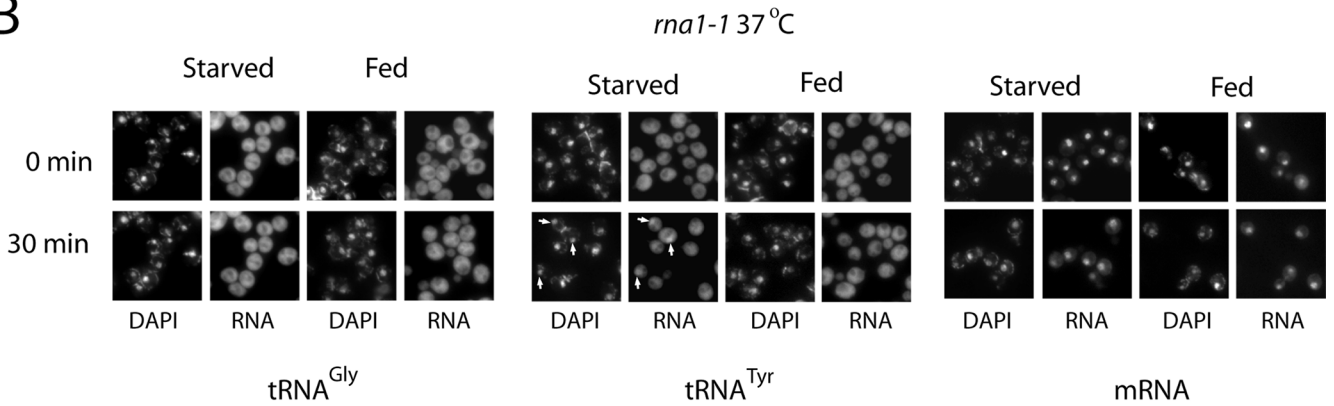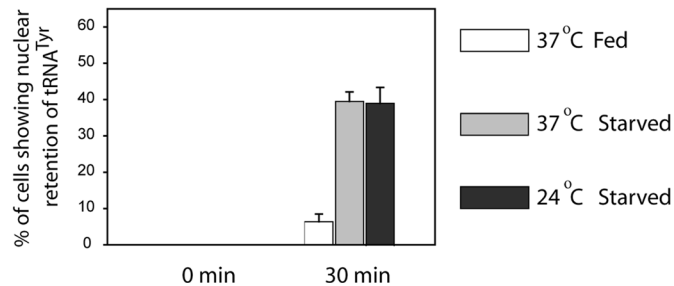
FIGURE 10: Loss of the function of Rna1p does not affect nuclear import of spliced tRNA during amino acid deprivation. (A) The temperature-sensitive *rna1–1* mutant strain was incubated at the permissive temperature (24°C) in complete synthetic medium or synthetic medium containing glucose and lacking amino acids for 30 min. (B) *rna1–1* was incubated at the nonpermissive temperature (37°C) in complete synthetic medium for 15 min to inactivate Rna1p and then switched to synthetic medium containing glucose and lacking amino acids for 30 min. The cellular location of tRNA^{Gly}, tRNA^{Tyr}, and mRNA was monitored by FISH. The DNA was visualized by DAPI staining and the arrows indicate nuclear accumulation of tRNA. (C) The percentage of cells showing nuclear retention of tRNA^{Tyr} in the *rna1–1* strain was quantified using several different fields from three independent experiments, and the average is plotted in a bar graph.

export. To investigate this possibility, the histidine, lysine, and leucine auxotrophic strain was deprived of amino acids, and the nuclear-cytoplasmic trafficking of tRNA^{Tyr}, tRNA^{Leu}, tRNA^{Gly}, and tRNA^{His} was monitored over a 6-h period (Figure 8). As observed before, starvation of the auxotrophic strain for 30 min results in nuclear accumulation of tRNA^{Tyr} (Figure 8A) and tRNA^{Leu} (Figure 8B) made from intron-containing pre-tRNAs. Furthermore, nuclear accumulation of both tRNAs was observed during the 6-h starvation

period. In contrast, nuclear accumulation of tRNA^{His} did not occur until the cells had been deprived of amino acids for 2 h, and this accumulation continued for the next 4 h (Figure 8D). Nuclear accumulation of tRNA^{Leu} was also shown previously to occur within 3 h of depriving a leucine auxotroph of leucine (Grosshans *et al.*, 2000). In contrast, nuclear accumulation of tRNA^{Gly} was not detected during the 6-h starvation period (Figure 8C). The later onset of nuclear accumulation of tRNA^{His} compared with that of the
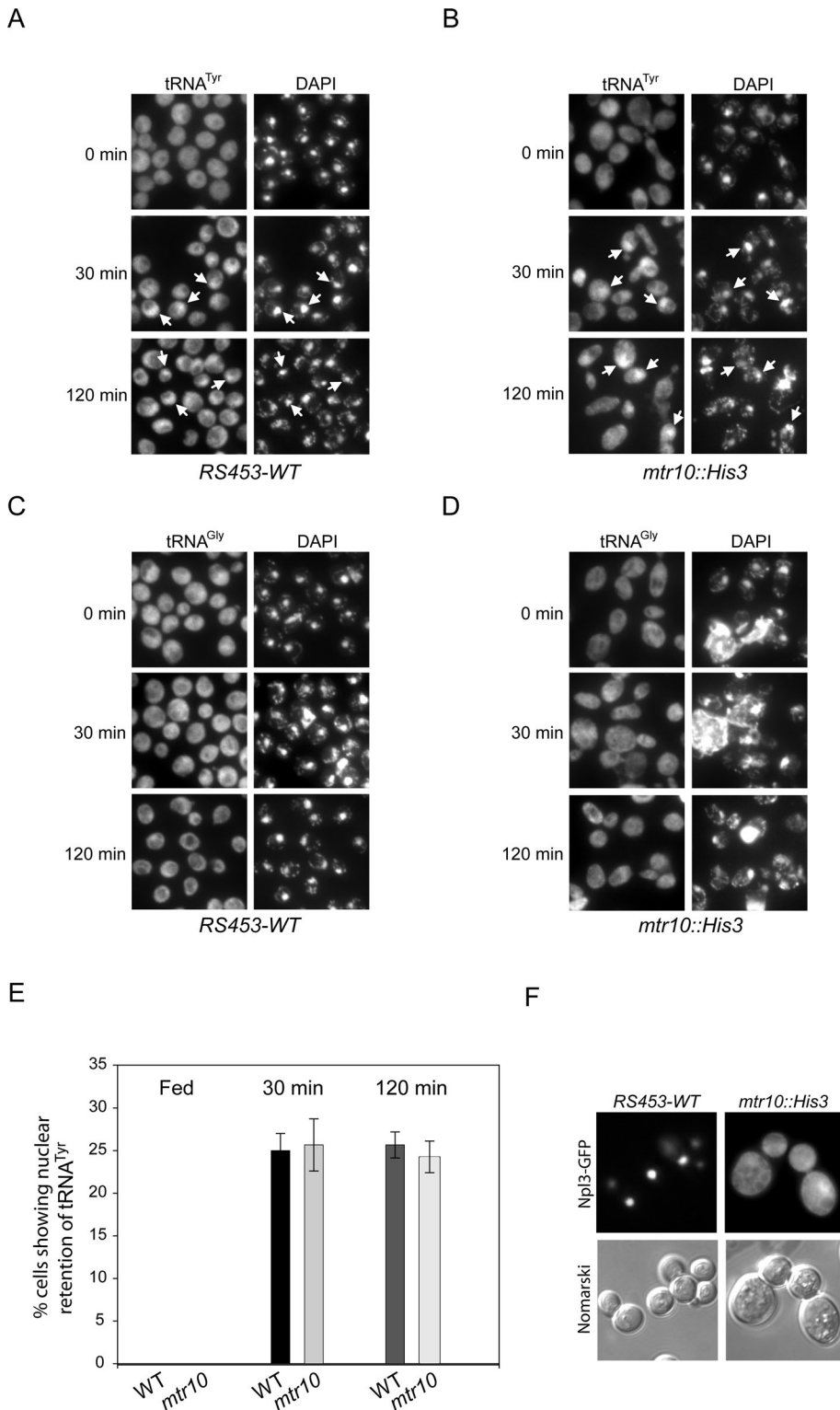
FIGURE 11: Mtr10p is not involved in nuclear import of cytoplasmic spliced tRNAs during amino acid deprivation. The wild-type *RS453* (A, C) and *mtr10::His3* (B, D) strains were incubated in synthetic media lacking amino acids but containing glucose for 30 min or 120 min. FISH was performed at the indicated time points to monitor the cellular location of mature tRNA^{Tyr} (A, B) and tRNA^{Gly} (C, D), and DNA was visualized using DAPI. The arrows indicate nuclear accumulated tRNAs that colocalize with DAPI staining. The percentage of cells showing nuclear retention of tRNA^{Tyr} in the *RS453* and *mtr10::His3* strains during the time course of amino acid starvation was quantified using three different fields from three independent experiments, and the average is plotted in a bar graph (E). To validate the *mtr10::His3* strain, the localization of Npl3p-GFP (F) was monitored in the wild-type *RS453* and *mtr10::His3* strains grown in synthetic complete media containing glucose by fluorescence microscopy.

spliced tRNA species suggests that lack of aminoacylation of tRNA^{His} is affecting the efficiency of nuclear export of the tRNA, which is consistent with reports demonstrating that nuclear tRNA aminoacylation is required for efficient export of tRNAs from the nucleus to the cytoplasm in *S. cerevisiae* (Grosshans *et al.*, 2000; Steiner-Mosonyi and Mangroo, 2004).

To test this notion further, the auxotrophic strain was starved of histidine only, and the cellular distribution of tRNA^{Tyr}, tRNA^{Leu}, tRNA^{His}, and tRNA^{Gly} was monitored by FISH (Figure 9). The analyses show that histidine starvation did not affect the distribution of tRNA^{Gly} during the time course of starvation (Figure 9C). As observed before, nuclear accumulation of tRNA^{His} was observed at 120 min of histidine starvation (Figure 9D). However, an effect on nuclear export of tRNA^{Tyr} (Figure 9A) and tRNA^{Leu} (Figure 9B) was observed after the cells had been starved of histidine for 240 min; this was only observed in ~12–15% of the cells analyzed. These data are also consistent with lack of aminoacylation of tRNA^{His} causing a reduction in the efficiency of nuclear export of the tRNA. Moreover, starvation of the cells for histidine also elicits a block in nuclear re-export of retrograded spliced tRNAs, but this response does not occur as quickly as when the cells are starved of all amino acids.

Collectively, the results demonstrate that, like mammalian, *K. lactis* and *S. pombe* cells, *S. cerevisiae* is also unable to transport cytoplasmic tRNAs to the nucleus in response to nutrient stress. Instead, the data strongly suggest that nuclear accumulation of retrograded spliced tRNAs during nutrient stress in *Saccharomyces* species is most likely due to a significant reduction in the efficiency of nuclear re-export of the retrograded tRNAs. This interpretation is consistent with data showing that nuclear import of spliced tRNAs occurs constitutively (Murthi *et al.*, 2010), and that overexpression of the nuclear tRNA export receptor Los1p restores nuclear export of tRNA^{Tyr} in cells starved of nitrogen (Pierce *et al.*, 2010). The data also suggest that the nuclear re-export pathway for retrograded spliced tRNAs, but not the pathway responsible for exporting mature tRNAs made from intronless pre-tRNAs, is most likely regulated by amino acid or nitrogen starvation. This is in agreement with results showing that inhibition of the TOR signaling pathway in unstarved cells using rapamycin causes nuclear accumulation of retrograded spliced tRNAs, but not tRNAs made from intronless pre-tRNAs (Pierce

et al., 2010). Also, a pathway responsible for nuclear re-export of retrograded spliced tRNAs, but not nuclear export of mature tRNAs made from intronless precursors, was recently identified in *S. cerevisiae* (Eswara et al., 2009).

Mtr10p and Rna1p do not function in nuclear import of cytoplasmic spliced tRNAs during nutrient stress

Previous studies have shown that inactivation of Rna1p, the protein that facilitates dissociation of the β-karyopherin-cargo-Gsp1p-GTP export complexes by activation of the GTPase activity of Gsp1p, blocks retrograde movement of a reporter tRNA from the cytoplasm to the nucleus in heterokaryons starved of amino acids (Shaheen and Hopper, 2005). This finding led others to suggest that the Gsp1pGTP/Gsp1pGDP cycle plays a role in this process and that a β-karyopherin may facilitate nuclear import of tRNA (Shaheen and Hopper, 2005) during nutrient stress. The β-karyopherin Mtr10p, which is involved in nuclear import of the RNA component of the *S. cerevisiae* telomerase, was shown to play a role in nuclear import of cytoplasmic tRNA during amino acid starvation (Shaheen and Hopper, 2005). In contrast, other studies found that inactivation of Rna1p had no effect on retrograde transport of tRNA from the cytoplasm to the nucleus in cells grown in compete medium (Takano et al., 2005). These data led to the suggestion that the Gsp1pGTP/Gsp1pGDP cycle and Mtr10p may facilitate nuclear import of tRNAs in response to nutrient-related stress (Shaheen and Hopper, 2005).

In view of our data suggesting that nutrient stress does not cause nuclear import of cytoplasmic tRNAs, we investigate whether loss of function of Rna1p blocks nuclear import of cytoplasmic spliced tRNAs during nutrient stress. For this analysis, the cellular distribution of tRNA^{Tyr} was monitored in an *rna1–1* temperature-sensitive strain deprived of amino acids (Figure 10). Starvation of *rna1–1* at the permissive temperature of amino acid for 30 min results in nuclear retention of tRNA^{Tyr} but not tRNA^{Gly} and mRNA (Figure 10A). Nuclear retention of tRNA^{Tyr} was detected in ~38% of the cells starved of amino acids (Figure 10C). Nuclear-cytoplasmic trafficking of tRNA^{Gly} was not affected in *rna1–1* preincubated at the nonpermissive temperature in complete synthetic medium for 15 min to inactivate Rna1p or when the mutant cells were incubated for an additional 30 min at 37°C (Figure 10B). Nuclear accumulation of tRNA^{Tyr} was also not observed when the *rna1–1* cells were preincubated at the nonpermissive temperature in complete synthetic medium for 15 min (Figure 10B). However, nuclear accumulation of tRNA^{Tyr} was observed in ~5% of the cells when the incubation was continued for an additional 30 min in complete synthetic medium at the nonpermissive temperature (Figure 10C). As reported previously, mRNA was retained in the nucleus of *rna1–1* preincubated for 15 min at the nonpermissive temperature in complete medium or when the fed cells were incubated for another 30 min at 37°C, confirming the loss of Rna1p function (Amberg et al., 1992) (Figure 10B). Similarly, nuclear accumulation of mRNA was detected when Rna1p-depleted cells were incubated in synthetic medium lacking amino acids for 30 min at the nonpermissive temperature, indicating that Rna1p remained inactive during the starvation period (Figure 10B). As observed before, amino acid starvation of Rna1p-depleted cells for 30 min at the nonpermissive temperature did not affect nuclear export of tRNA^{Gly}. Nuclear accumulation of tRNA^{Gly}, however, was observed when starved and unstarved *rna1–1* cells were incubated at the nonpermissive temperature for 3 h, which is consistent with genetic and biochemical data indicating that Rna1p is required for nuclear export

of tRNAs in *S. cerevisiae* (data not shown) (Hellmuth et al., 1998; Sarkar and Hopper, 1998; Eswara et al., 2009). Interestingly, starvation of Rna1p-depleted cells of amino acids for as little as 30 min at the nonpermissive temperature resulted in nuclear retention of tRNA^{Tyr} (Figure 10B). The percentage of starved *rna1–1* cells showing nuclear tRNA^{Tyr} accumulation at the nonpermissive temperature is comparable to that for *rna1–1* cells starved at the permissive temperature (Figure 10C). Moreover, loss of Rna1p function also does not block nuclear import of spliced tRNAs during nitrogen starvation (data not shown). These data, in contrast to previous studies, suggest that loss of Rna1p function does not block nuclear import of spliced tRNAs during amino acid starvation and that the Gsp1pGTP/Gsp1pGDP cycle may not be involved in nuclear import of cytoplasmic spliced tRNAs in response to nutrient stress.

On the basis of the finding that the Gsp1pGTP/Gsp1pGDP cycle does not play a role in nuclear tRNA import, we tested the involvement of Mtr10p in this process during nutrient stress. The analysis involved monitoring the effect of amino acid starvation on nuclear-cytoplasmic trafficking of tRNA^{Tyr} and tRNA^{Gly} in a *mtr10* mutant strain (Figure 11). FISH analyses show that the cellular distribution of both tRNA species was not affected in the unstarved wild-type (Figure 11, A and C) and *mtr10* (Figure 11, B and D) strains. Starvation of both the wild-type and *mtr10* strains of amino acids for 30 or 120 min resulted in nuclear accumulation of tRNA^{Tyr} (Figure 11, A and B) but not tRNA^{Gly} (Figure 11, C and D). Nuclear accumulation of tRNA^{Tyr} was detected in the same percentage of wild-type (Figure 11A) and *mtr10* (Figure 11B) cells starved of amino acid for 30 and 120 min (Figure 11E). Consistent with previous studies, loss of function of Mtr10p resulted in a loss of nuclear import of Npl3-GFP (Figure 11F), demonstrating that the *mtr10* strain behaves as reported (Senger et al., 1998). As expected, nuclear import of Npl3-GFP was observed in the wild-type strain (Figure 11F). These data, unlike studies reported, suggest that Mtr10p is also not involved in nuclear import of spliced tRNAs during nutrient stress. Moreover, the data suggest that a β-karyopherin may not facilitate nuclear import of spliced tRNAs during nutrient deprivation.

CONCLUSIONS

The results of this study demonstrate that evolutionarily diverse eukaryotic organisms, including *S. cerevisiae*, do not use nuclear import of cytoplasmic tRNAs as a mechanism to facilitate nutrient-related regulation of gene expression and cell growth. However, it appears that retrograde movement of spliced tRNAs from the cytoplasm to the nucleus is limited to yeasts of the genus *Saccharomyces* and re-export of these tRNA species are affected by nutrient stress. The reason why only species of *Saccharomyces*, but not other yeasts and higher eukaryotes, have acquired a nuclear tRNA import process for spliced tRNAs during evolution is unclear, although this may in part be related to the location of the tRNA-splicing machinery. Furthermore, it is not known how amino acid or nitrogen deprivation affects nuclear re-export of retrograded tRNAs made from intron-containing precursors or why nuclear re-export of retrograded spliced tRNAs is regulated differently from nuclear export of tRNAs made from intronless precursors in *Saccharomyces* species. The regulation of nuclear re-export of retrograded spliced tRNAs by amino acid or nitrogen availability appears to involve the TOR signaling pathway (Pierce et al., 2010). Moreover, recent studies suggest that the functions of the nuclear tRNA export receptors may be regulated, as overexpression of the nuclear tRNA export receptor Los1p restored nuclear re-export of retrograded spliced tRNA^{Tyr} in *S. cerevisiae* starved of nitrogen

(Pierce *et al.*, 2010). Finally, the finding that several conditions affect nuclear tRNA export in *Saccharomyces* species but not in other eukaryotes suggests that there are fundamental mechanistic differences between the *Saccharomyces* nuclear tRNA export process and that of other organisms.

MATERIALS AND METHODS

Materials
The HeLa, HEK293, and rat hepatoma H4IIE cell lines were obtained from the American Type Culture Collection (Manassas, VA). *S. bayanus* and *K. lactis* were obtained from G. van der Merwe (University of Guelph, Canada), and *S. paradoxus* was provided by E. Louis (Institute of Genetics, University of Nottingham, UK). The genotypes of the yeast strains used in this study are provided in Table 1. The sequences of the oligonucleotides used for FISH, antibodies, sources of plasmids, and Northern blot analyses are provided in Supplemental Materials.

Preparation of cell extract from mammalian cells
HeLa, HEK293, and rat hepatoma H4IIE cells were grown on 10-cm^2 dishes until 80% confluent. The cells were washed with 1× PBS, pH 7.4, and incubated at 37°C with serum-free DMEM lacking all amino acids, or Lys, Arg, Leu, and Gln for 4 h, serum-free DMEM containing 10 mM NaN$_3$ for 2.5 h, serum-free DMEM without glucose for 4 h, 1× PBS, pH 7.4, containing 1000 mg/l glucose for 2.5 h, or Ham's F-12 nutrient media lacking Met, Lys, Leu, and Gln (Sigma, St. Louis, MO) for 2.5 h. The cells were washed with 1× PBS and lifted with TrypLE express (Invitrogen, Carlsbad, CA). The cells were collected by centrifugation, resuspended in NP-40 buffer (10 mM Tris, pH 7.5, containing 150 mM NaCl, 1 mM Na$_3$VO$_4$, 10 mM NaF, 2 mM EDTA, and 1% NP-40 [vol/vol]) containing 1× PIN cocktail, and incubated on ice for 10 min. The cells were lysed by vortexing and centrifuged at 15,000 rpm at 4°C for 10 min. The cell extracts were subjected to SDS–PAGE on a 15% polyacrylamide gel, and Western blot analysis was performed with α-4E-BP1 and α-actin.

Analysis of the nuclear-cytoplasmic distribution of tRNA in mammalian cells by FISH using Cy3-labeled oligonucleotides
Cells were fixed as described at http://www.singerlab.org/protocols and by Sarkar and Hopper (1998) and Chafe and Mangroo (2010). Fixed cells were then rinsed with 1× PBS and permeabilized in 1× PBS containing 0.5% Triton X-100 (vol/vol) for 10 min on ice. The cells were rehydrated for 5 min in 4× SSC, 50% formamide (vol/vol) and hybridized overnight at 37°C with 5–40 pmol of Cy3-labeled probe in 4× SSC (1× SSC = 150 mM NaCl, 15 mM sodium citrate, pH 7.0), 50% formamide (vol/vol), 10% dextran sulfate (wt/vol), 0.2% bovine serum albumin (wt/vol), 125 µg/ml *Escherichia coli* tRNA, 500 µg/ml sheared salmon sperm DNA, and 2 mM vanadyl ribonucleoside complex in a volume of 30 µl. Cells were washed at room temperature with 4×, 2×, 1×, and 0.5× SSC containing 50% formamide (vol/vol), then stained with 1 µg/ml DAPI, washed three times with 1× PBS, and mounted using Dako fluorescent mounting media. The human oligonucleotides used were designed using the tRNAscan-SE database (Lowe and Eddy, 1997), and the rat oligonucleotide was described previously (Shaheen *et al.*, 2007).

Detection of nuclear-cytoplasmic distribution of tRNA in mammalian cells using DIG-UTP-labeled oligonucleotide
The tRNA^{Lys} probe was labeled at the 3′ end using the DIG 3′ end-labeling kit (Roche, Quebec, Canada) as per the manufacturer's instructions. Cells were prepared for FISH as described above, and

the DIG-UTP-labeled tRNA^{Lys} probe (10–40 pmol) was added to the hybridization solution in a final volume of 30 µl and incubated overnight in a humid chamber at 37°C. Cells were then washed with 4×, 2×, 1×, and 0.5× SSC containing 50% formamide (vol/vol) for 5 min each. Following the final wash, the cells were washed with 1× PBS and then blocked with 5% skim milk powder (wt/vol) in 1× PBS for 1 h at room temperature. The cells were then incubated with fluorescein-conjugated anti-DIG antibodies (1:50 dilution) for 1 h at 37°C in PBS-T (1× PBS, containing 0.1% Tween 20). The cells were then washed with PBS-T, DAPI stained, and mounted using Dako.

Nuclear-cytoplasmic distribution of tRNA in yeast
The *Saccharomyces* species and *K. lactis* were grown in complete synthetic media containing 2% glucose (wt/vol) to midlogarithmic phase at 30°C. Then, 50 ml of culture was washed twice with sterile water and incubated in synthetic media containing glucose and lacking nitrogen or amino acids. At the specified time, the cells were processed for FISH to detect nuclear-cytoplasmic distribution of tRNA as described (Grosshans *et al.*, 2000; Steiner-Mosonyi and Mangroo, 2004).

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